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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We previously demonstrated that breast cancer cells constitutively express high levels of nuclear NF-κB/Rel, which correlated with decreased stability of IκB inhibitory proteins. Our objective is to elucidate the mechanisms leading to IκB turnover and thereby aberrant NF-κB activation in breast cancer. Activation of NF-κB has clearly been shown to be dependent upon the rate of IκB turnover which is regulated by 1) the two kinase components (IKKα and IKKβ) of the IκB kinase complex (IKK), and 2) the protein kinase CK2. The relative contribution of these kinases depends upon signal and cell context. We assessed the expression level and kinase activity of CK2 and the IKKα and IKKβ in human and mouse breast cancer cell lines, as well primary breast cancer specimens from patients and from transgenic mice. We also tested the hypothesis that CK2 and IKK kinases play an important role in the growth, survival and transformed phenotype of breast cancer cells through NF-κB activation. Overall, these studies should contribute to the understanding of the signaling mechanisms involved in the constitutive activation of NF-κB in mammary tumors and may help define novel therapeutic targets for the treatment of breast cancer.

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Introduction

The Nuclear Factor-κB (NF-κB)/Rel transcription factors regulates genes that control cell proliferation, survival, and transformed phenotype. While in normal breast epithelial cells NF-κB/Rel proteins are mainly sequestered in the cytoplasm bound to one of the specific inhibitory IκB proteins, we and others previously demonstrated their aberrant activation in breast cancer (1, 2).

We proposed to test the hypothesis that the signaling pathway(s) regulating the rate of IκB-α turnover is altered in breast cancer cells, leading to aberrant activation of NF-κB/Rel. Much progress has been made in elucidating the kinases that regulate IκB-α stability. These appear to function via phosphorylation of N- or C-terminal sites of IκB-α. A variety of agents that activate NF-κB, e.g., TNF-α and IL-1, mediate degradation of IκB-α via a canonical pathway involving phosphorylation of IκB on two N-terminal serine residues by a large multi-subunit complex comprised of the two IκB kinases IKKα and IKKβ (3). This phosphorylation is followed by ubiquitination and rapid proteasome-mediated degradation of IκB, allowing for translocation of free NF-κB to the nucleus. In addition, phosphorylation of IκB by protein kinase CK2 (formerly casein kinase II) has been implicated in basal IκB-α degradation (4-6). CK2-mediated phosphorylation of IκB-α occurs preferentially at Ser-283, Ser-289, Thr-291 and Ser-293 within the C-terminal PEST domain, and mutations of these sites prolong IκB-α half-life (6, 7). These findings supported the rationale for studying the possible involvement of both kinases in breast carcinogenesis.

Research accomplishment to date

Aim 1. Determine the levels of IKK and CK2 kinases in breast cancer cells

We recently showed that elevated levels of NF-κB in the D3-1 and BP-1 lines, derived by *in vitro* transformation of MCF-10F human breast epithelial cells by 7,12-dimethylbenz(*a*)anthracene (DMBA)- and benzo[*a*]pyrene (BaP), respectively, correlated with a decrease in the half-life of IκB-α protein (8).

To assess the mechanisms of this aberrant activation of NF-κB expression, here we measured expression levels of major kinases implicated in IκB-α stability, namely IKKα, IKKβ and protein kinase CK2 in those cell lines. We also studied other human and mouse breast cancer cell lines e.g. Hs578T human breast cancer cells, MMTV-Her-2/neu-derived NF639 mouse breast cancer cells, and NMuMG mouse untransformed breast epithelial cells. We observed that levels of IKKα and IKKβ were unchanged in breast cancer cells compared to breast epithelial cells, suggesting changes in the activation levels of those kinases (see below). In contrast, we observed increased expression levels of CK2 in human (Fig. 1A) and mouse (Fig. 1B) breast cancer cells compared to epithelial cells. This is consistent with previous reports showing that CK2 levels are elevated in proliferating cells, and also in tumor cells and neoplastic tissues of various origins (reviewed in (9)).

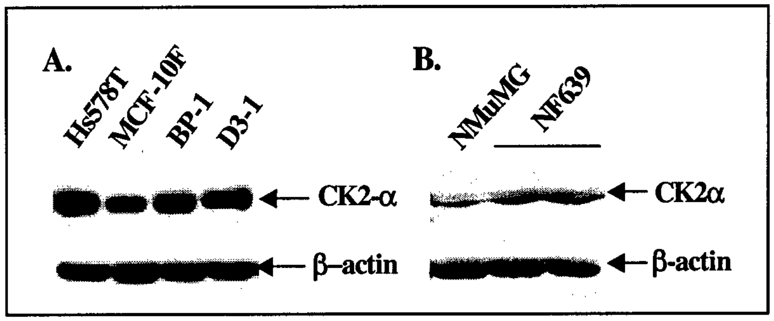

Fig. 1. CK2α expression in human and mouse breast cancer cell lines. WCEs were prepared from A) exponentially growing Hs578T, BP-1 and D3-1 human breast cancer cell lines and MCF-10F untransformed human breast epithelial cells and B) from two independent cultures of NF639, and NMuMG untransformed mouse breast epithelial cells at 70% confluence. Samples (80 μg) were subjected to immunoblot analysis for CK2α and β-actin levels.

Aim 2. Evaluate activities of the IKK and CK2 kinases in breast cancer cells.

To analyze IKK kinase activity, samples of whole cell extracts containing equal amounts of proteins were immunoprecipitated with an IKKβ kinase specific antibody. One third of the immunoprecipitated material was used in *in vitro* phosphorylation assays with full length WT IκB-α fusion protein (GST-wtIκB-α) as substrate, and protein was labeled with [γ-^{32}P] ATP (Fig. 2, upper panel). The remainder was subjected to immunoblotting for IKKα protein as indicated (Fig. 2, bottom panel). Results obtained indicated that Hs578T, BP-1 and D3-1 breast cancer cell lines displayed an IκB-α kinase activity directed by IKKα (Fig. 2) and IKKβ (data not shown) increased compared to MCF-10F cells. IKK activity was specific for Ser-32 and Ser-36 of IκB-α because replacement with alanine at both sites in the GST-IκB-α substrate eliminated phosphorylation.

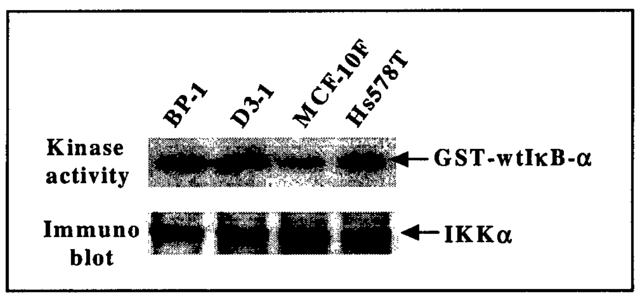

Fig. 2. Analysis of IKKα kinase activities in human breast cancer cell lines. Whole cell extracts were prepared from Hs578T, D3-1, and BP-1 breast cancer cells and from untransformed MCF-10F breast epithelial cells. Equal amounts (150 μg) were immunoprecipitated with an antibody against IKKα. Samples (one third total) were subjected to a kinase assay using GST-wtIκB-α as substrate (top panel), while the remainder (two thirds) were subjected to immunoblotting for IKKα protein.

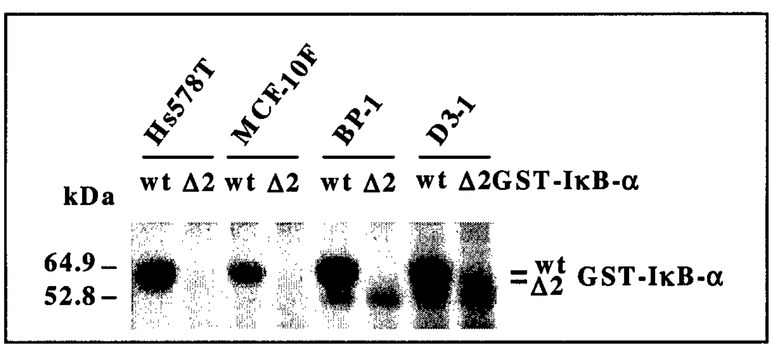

Fig. 3. CK2 activity is elevated in breast cancer cell lines. WCEs were prepared from cultures of Hs578T, D3-1 and BP-1 breast cancer cells and untransformed MCF-10F cells at 70% confluence. A) CK2 activity. Samples (10 μg) were subjected to a CK2 kinase assay, as above, using as substrate either GST-wtIκB-α (wt) or GST-Δ2IκB-α (Δ2, with a deletion of amino acids 269-317 in the C-terminal PEST domain of IκB-α).

A similar analysis was next performed for CK2, using as a substrate either GST-IκB-α or a CK2-specific phosphorylation peptide. CK2 kinase activity was significantly lower in the untransformed MCF-10F cells compared to all three breast cancer cell lines (Fig. 3).

We next asked whether these three IκB kinases are activated in primary human breast cancer specimens and whether NF-κB induction correlates with kinase activation. Two sets of primary human breast cancer tissue specimens were obtained from patients undergoing surgery for treatment of breast cancer with approval of the Institutional Review Board of Boston Medical Center. Nuclear extracts were prepared from frozen breast tumors and used for NF-κB activity by EMSA and immunoblot analysis. Since potential contamination with hematopoietic cells could significantly affect the analysis, our strategy was to also test for such contamination using a binding assay for PU.1 and TCF-1, which are present in B lymphocytes, neutrophils, mast or myeloid cells and T cells. Table 1 presents the findings for every PU.1- and TCF-1-negative sample from the two sets analyzed. Cytoplasmic extracts from tumors of these patients were then tested for CK2 kinase activity using GST-wtIκB-α as substrate. Three of the specimens displayed low levels of CK2 activity (231 to 415 densitometry units), while the remaining specimens had either modestly increased (758 to 1,106 densitometry units) or substantially elevated levels (1,809 to 6,143 densitometry units) (Table 1). The three specimens with low CK2 also displayed low or minimally elevated NF-κB binding. Six samples displayed elevated levels of CK2 and NF-κB binding, while only one specimen (#6885) showed high IκB-α CK2 kinase activity without detectable high NF-κB nuclear activity. Activities of the IKKα and IKKβ kinases were evaluated following immunoprecipitation with their specific antibodies (Table 1). Tumor samples #6731 and #8364 exhibited modestly increased levels of IκB-α kinase activity directed by IKKα, and increased NF-κB binding activity. In the analysis of IKKβ kinase activity most of the samples yielded values between approximately 100 and 300 densitometry units, however, cytosolic extracts from 3 specimens (#8361, #8364, #8385) displayed greatly elevated IKKβ activity (1,164, 1,395 and 980 densitometry units, respectively). These 3 specimens all displayed high NF-κB binding activity.

Table 1. IKK and CK2 kinase activity in primary human breast specimens. Table presents results observed with two groups of 6 and 10 primary human breast specimens. Only data from PU.1- and TCF-1-negative tumors are showm. Data were normalized using the same cell extracts from WEHI 231 B cell in every assay.

						kinase activity		
Patient	**Tumor type**	**ER/PR levels (fmol/mg)**	**NF-kB binding***	**RelA***	**c-Rel***	**IKKa***	**IKKb***	**CK2***
6731**	Right breast mass	026 / 162	4 868	1 137	687	432	114	1 809
6712**	Right infiltrating ductal cancer	006 / 000	3 939	2 578	nd	129	178	2 446
6885	Not avalaible	002 / 010	114	-	-	230	316	6 143
8357	Right invasive lobular cancer	000 / 000	970	-	-	-	ND	341
8359	Ductal cancer	001 / 004	646	-	-	182	276	415
8360	Left infiltrating ductal cancer	051 / 035	344	-	-	-	294	231
8361	Right breast mass	022 / 006	6 022	4 764	74	133	1 164	1 106
8364	Not avalaible	123 / 372	1 866	1 713	-	355	1 395	819
8385	Metastatic cancer	018 / 011	2 840	2 938	652	247	980	758
6698	Left breast mass	367 / 033	3 689	-'***	-	137	243	3 902

* Densitometry, arbitrary units

**from the first series of patients

***, no expression/activity detected; nd, not done

Thus breast cancers display activation of either CK2, IKKα, IKKβ, or various combinations of these kinases, which correlate and are therefore likely responsible for the aberrant NF-κB activation in breast cancer cell lines as well as primary tumors.

***Aim 3*. Elucidate the functional roles of IKK and CK2 kinases in breast cancer cell proliferation and survival.**

3.1. Expression vectors used for the study.

We obtained or constructed vectors necessary for CK2, IKKα, IKKβ, and wild type (WT) or mutant IκB proteins. The murine CK2α cDNA (10) was excised from the pcDNA3.0 vector (Invitrogen Life Technologies) by Bam HI digestion and subcloned into the Bam HI site of the pBabe-puro ecotropic retroviral vector (11), yielding pBabe-puro-CK2α. The pRc/CMV2 HA-tagged CK2α (pZW2), pRc/CMV2-HA-CK2α K68M (pGP8), pRc/CMV2-HA-CK2α' (pGP3), pRc/CMV2-HA-CK2α' K69M (pGP18) vectors, and the backbone empty vector were kindly provided by D.W. Litchfield (University of Western Ontario, Ontario, Canada, Ref (12)). The CK2α K68M or CK2α' K69M mutants contain a single point mutation in the kinase domain of CK2α and CK2α' catalytic subunits, respectively, and are devoid of kinase activity (12). The pRC-βactin-IKKαSS/AA vector, which expresses a phosphorylation-defective mutant IKKαSS/AA that functions as a dominant negative version of IKKα, and the parental pRC-βactin vector were provided by our collaborator F. Mercurio (3). The pRC-βactin-IKKα S176,S180/AA vector insert was subcloned into the pcDNA3 vector yielding, pcDNA3-IKKα S176,S180/AA . The plasmids pCMV-IKKβ S177,S181/AA and pCMV-IKKβSS/EE allowing expression of a dominant negative mutant flag-tagged IKKβ and a constitutively active flag-tagged IKKβ respectively, were also obtained from F. Mercurio (3). The human pSVK3-IκB-α WT, S32,S36/AA (2N) mutant, S283,T291,T299/AAA (3C) mutant, and S32,S36,S283,T291,T299/AAAAA (2N3C) mutant were kindly obtained from J. Hiscott (Institut Lady Davis de Recherches Medicales, Montreal, Canada, Ref (5)), and subcloned into the EcoRI site of the pBabe-puro ecotropic retroviral vector.

3.2. Introduction of retroviral and/or expression vectors in mammary epithelial cells or mammary tumor cells and selection of stable cell lines.

Phoenix packaging cells were used for generation of retrovirus, which were selected with 500 μg/ml hygromycin to increase Gag and Pol viral protein expression. Briefly, 80% confluent Phoenix cells were transfected with pBabe-puro-CK2α, pBabe-IκB-α WT or mutants, or pBabe-puro along with an Env-expressing vector. After 24 h, the medium was changed and cells were incubated for another 24 h at 32°C to increase retrovirus half-life. Supernatants containing retrovirus were then harvested, filtered and transferred on target cells (NIH 3T3 fibroblasts or NF639 mouse breast cancer cells) in the presence of 2 μg/ml polybrene. After 24 h, infected cells were washed, selected with complete medium plus 4 μg/ml puromycin for 4 days, and expanded in medium containing 1 μg/ml puromycin. Single cell clones were isolated by limiting dilution. As a positive control, cells were infected with the pBabe-puro-GFP retrovirus, indicating more than 90% efficiency in retroviral infection of NIH 3T3 cells.

To establish stable transfectants expressing ectopic WT or mutant CK2α, breast cancer cells (Hs578T and NF639 cells) were transfected with appropriate pRc-CMV constructs and selected with 600 μg/ml geneticin for 10 days, and then grown in the presence of 100 μg/ml geneticin. Alternatively, cells were co-transfected with 1 μg of the pGKpuro selection plasmid, selected with 4 μg/ml puromycin for 4 days, and then grown in the presence of 1 μg/ml puromycin.

3.3. Analysis of the effects of inhibition or overexpression of IKK and CK2 kinases in NF-κB binding and transcriptional activity, IκB-α phosphorylation, cell growth survival and transformed phenotype.

We first analyzed effects of inhibition of IKK activity upon stable or transient expression of dominant negative kinases IKKα SS/AA, IKKβ SS/AA in Hs578T breast cancer cells. Expression of those mutants inhibited NF-κB transcriptional activity (Fig. 4A). Furthermore, inactivation of the IκB kinase complex in Hs578T cells via expression of a C-terminal mutant and dominant negative IKKγ/NEMO/IKKAP1 reduced soft agar colony growth (Fig. 4B).

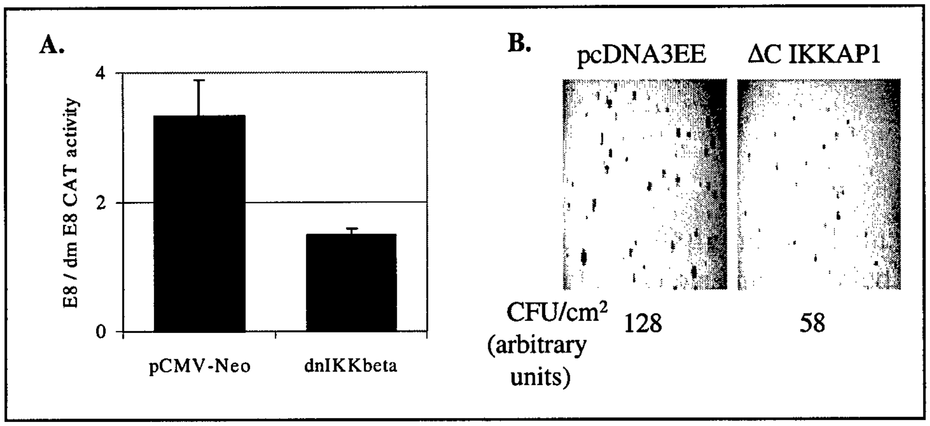

Fig. 4. Inhibition of IKK reduces NF-κB activity and anchorage-independent growth of Hs578T human breast cancer cells. A) Cells were transiently transfected, in triplicate, with 1 μg of wt E8-CAT (E8) or dmE8-CAT (dmE8) NF-κB element-thymidine kinase (TK) promoter-chloramphenicol acetyltransferase (CAT) containing 2 copies of either the wild type or mutant NF-κB element from upstream of the *c-myc* promoter (13), 0.5 μg SV40-βgal in the presence of 1 μg of pCMV-Neo parental or pCMV-IKKβSS/AA vector, expressing dominant negative IKKβ protein (dnIKKbeta). After 24 h, cultures were harvested, and samples, normalized for βgal activity, assayed for CAT activity. The values for E8-CAT activity are represented as fold induction over dmE8-CAT activity. B) Cells were transfected, in triplicate, with parental pcDNA3EE vector or with ΔC IKKAP1 vector, and assessed for growth in soft agar. The number of colonies are shown below.

We next investigated the role of protein kinase CK2 in oncogene-induced NF-κB activation in breast cancer cells. The Her-2/neu oncogene, the second member of the epidermal growth factor (EGF) receptor family, encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu in ~30% of breast cancers is associated with poor overall survival. Recently, we found that Her-2/neu activates NF-κB via a phosphatidylinositol 3 kinase (PI3-K)-Akt kinase signaling pathway in MMTV-Her-2/neu NF639 mouse breast cancer cells (14). Surprisingly, the IKK kinase complex, implicated in proteasome-mediated degradation of IκB-α and activation of NF-κB via the canonical pathway, was not activated in these cells. Degradation of IκB-α in those cells was mediated via calpain, which in B cells is facilitated by phosphorylation of IκB-α by the protein kinase CK2 (14). Here, we reported that inhibition of CK2 blocks Her-2/neu-mediated activation of NF-κB. NF639 breast cancer cells stably expressing CK2α or CK2α' kinase-inactive mutants displayed decreased NF-κB binding (Fig. 5).

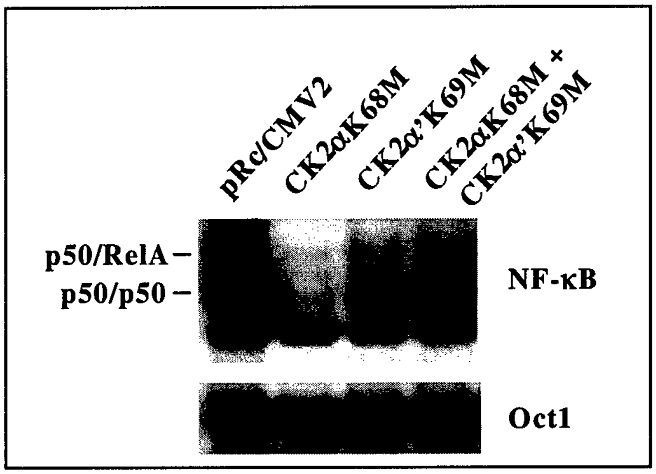

Fig. 5. Expression of kinase-inactive CK2α or CK2α' subunits inhibits constitutive NF-κB binding activity in NF639 breast cancer cells. Cells were transfected with the indicated plasmid expression vectors, and mixed populations of stably transfected cells selected with puromycin. Nuclear extracts were prepared and samples (5 μg) subjected to EMSA using as probes oligonucleotides containing either NF-κB or Oct1 binding elements. The position of the p50/p50 and p50/RelA complexes, identified previously (14), are as indicated. Similar results were observed with two additional independent sets of NF639 cells stably transfected with CK2 mutant constructs.

Interestingly, the inhibition in NF-κB activity in cells with reduced CK2 activity was associated with a reduced ability to grow in soft agar (Fig 6B), as well as increased sensitivity to TNF-α killing (Fig. 6A). Similarly, CK2 kinase inactive subunits inhibited NF-κB activity in Hs578T human breast cancer cells (data not shown), which also display elevated CK2 activity.

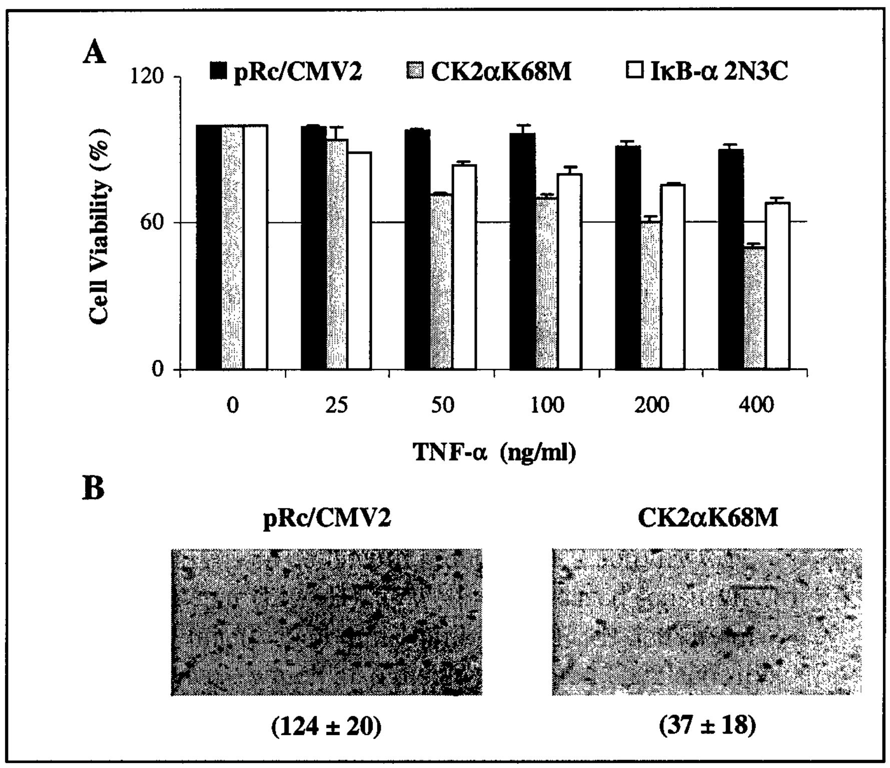

Fig. 6. Expression of kinase-inactive CK2α subunit increases susceptibility to TNF-α-induced apoptosis and reduces anchorage-independent growth of NF639 breast cancer cells. A) TNF-α-induced cell death. Mixed population of NF639 cells stably transfected with HA-CK2α K68M, parental pRc/CMV2 or IκB-α 2N3C super-repressor plasmid expression vectors were plated at 2 x 10^3 in 96-well plates. After 24 h, cells were treated with recombinant human TNF-α in the presence of 30 μg/ml cycloheximide. After 16 h, cell viability was evaluated by the MTS cell proliferation assay. Results are expressed as percentage of viable cells (absorbance at 490 nm normalized to absorbance of cultures treated with cycloheximide alone). B) Soft agar assay. The indicated cells were plated, in triplicate, at 3 x 10^3/ml in top plugs consisting of complete medium and 0.4% agarose. After 18 days, the numbers of foci were scored, and pictures taken with a Nikon camera (magnification x1.5). Values of colony number per high power field are given below.

In NIH 3T3 fibroblasts, which express low basal NF-κB and CK2 activities, overexpression of CK2 by retroviral gene delivery led to induction of classical NF-κB (i.e. p50/RelA, Fig. 7A and data not shown) and increased IκB-α turnover (Fig. 7B). Thus, CK2 plays an important role in Her-2/neu signaling, promoting IκB-α degradation and thereby NF-κB activation. Furthermore, since ectopic CK2 activity appears sufficient to induce NF-κB, the elevated CK2 activity observed in many primary human breast cancers likely plays a role in aberrant activation of NF-κB, and therefore represents a potential therapeutic target.

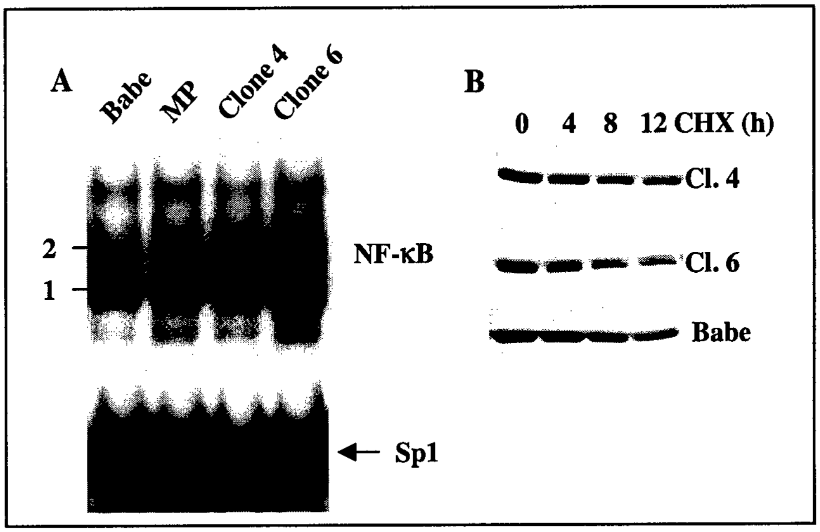

Fig. 7. Overexpression of CK2α subunit results in increased NF-κB activity and higher rate of IκB-α degradation and activity in NIH 3T3 cells. Cells were infected with recombinant retrovirus for pBabe (Babe) or pBabe-CK2α vectors. A mixed population of Babe-CK2α (MP) cells and two clones (#4 and #6) were selected in puromycin. A) NF-κB binding activity. Nuclear extracts were prepared from the indicated cells, and samples (5 μg) subjected to EMSA with oligonucleotides specific for NF-κB and Sp1. The positions of the two major NF-κB complexes are indicated as band 1 and band 2. B) IκB-α half-life. The indicated cells, in exponential growth, were treated with 50 μg/ml cycloheximide (CHX), and WCEs prepared at 0, 4, 8 or 12 h. Samples (50 μg) were subjected to immunoblot analysis for IκB-α.

Reportable outcomes

Constructs:

- pBabe-puro-CK2α WT, pBabe-CK2α' WT retroviral vectors
- pBabe-puro-IκB-α WT, S32,S36/AA (2N) mutant, S283,T291,T299/AAA (3C) mutant, and S32,S36,S283,T291,T299/AAAAA (2N3C) mutant retroviral vectors
- pcDNA3-IKKα S176,S180/AA plasmid expression vector

Manuscript:

Romieu-Mourez R. Landesman-Bollag E., Seldin DC., Sonenshein GE. "Protein kinase CK2 promotes aberrant activation of NF-κB, transformed phenotype and survival of breast cancer cells", Cancer Res., 2002, 62: 6770-6778.

Abstract:

Romieu-Mourez R., . Kim DW., Shin S., Landesman-Bollag E., Seldin DC., and Sonenshein GE. "c-Rel or activation of NF-kB by protein kinase CK2 promote breast cancer" Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Sep. 25-28, 2002, Orlando, Florida (poster presentation).

Conclusion

To understand the mechanism of aberrant NF-κB/Rel expression in breast cancers, we evaluated the activation of the major kinases implicated in regulation of IκB stability, namely IKKα, IKKβ, and protein kinase CK2. Human and mouse breast cancer cell lines and primary breast tissues from patients display high levels of IKKα, IKKβ, and CK2 activity. Inhibition of IKK activity decreased NF-κB/Rel activity and soft agar colony growth in breast cancer cells. Furthermore, primary human breast cancer specimens, that displayed aberrant constitutive expression of NF-κB/Rel, were found to exhibit increased CK2 and/or IKK kinase activity. We also reported that CK2 regulate the basal NF-κB activity in various cell lines. In addition, CK2 plays a role in Her-2/neu signaling, promoting IκB-α degradation and NF-κB activation. In cells expressing low basal NF-κB and CK2 activities, such as NIH 3T3 fibroblasts, overexpression of CK2 increased IκB-α turnover and induced classical NF-κB. Thus, the aberrant expression of CK2 or IKK kinases promotes increased nuclear levels of NF-κB/Rel and transformation of breast cancer cells. These observations suggest these kinases play a similar role in an intracellular signaling pathway that leads to the elevated NF-κB/Rel levels seen in primary human mammary tumors, and therefore represent potential therapeutic targets in the treatment of patients with breast cancer.

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Appendices

Please find enclosed a reprint of the manuscript by Romieu-Mourez R. Landesman-Bollag E., Seldin DC., Sonenshein GE. "Protein kinase CK2 promotes aberrant activation of NF-κB, transformed phenotype and survival of breast cancer cells", Cancer Res., 2002, 62: 6770-6778.

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Protein Kinase CK2 Promotes Aberrant Activation of Nuclear Factor-κB, Transformed Phenotype, and Survival of Breast Cancer Cells[1]

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ABSTRACT

The *Her-2/neu* oncogene, the second member of the epidermal growth factor (EGF) receptor family, encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu in ~30% of breast cancers is associated with poor overall survival. Recently, we have found that Her-2/neu activates nuclear factor (NF)-κB via a phosphatidylinositol 3 kinase (PI3-K)-Akt kinase signaling pathway in mouse mammary tumor virus (MMTV)-Her-2/neu NF639 mouse breast cancer cells. Surprisingly, the IκB kinase (IKK) kinase complex, implicated in proteasome-mediated degradation of IκB-α and activation of NF-κB via the canonical pathway, was not activated in these cells. Degradation of IκB-α was mediated via calpain, which in B cells is facilitated by phosphorylation of IκB-α by the protein kinase CK2. Here, we report that the inhibition of CK2 blocks Her-2/neu-mediated activation of NF-κB. NF639 breast cancer cells, stably expressing CK2α or CK2α′ kinase-inactive mutants, displayed decreased NF-κB binding and reduced ability to grow in soft agar, as well as increased sensitivity to tumor necrosis factor (TNF)-α killing. Similarly, CK2 kinase-inactive subunits inhibited NF-κB activity in Hs578T human breast cancer cells, which also display elevated CK2 activity. In NIH 3T3 fibroblasts, which express low basal NF-κB and CK2 activities, overexpression of CK2 by retroviral gene delivery led to increased IκB-α turnover and the induction of classical NF-κB (p50/RelA). Thus, CK2 plays an important role in Her-2/neu signaling, promoting IκB-α degradation and, thereby, NF-κB activation. Furthermore, because ectopic CK2 activity appears sufficient to induce NF-κB, the elevated CK2 activity observed in many primary human breast cancers likely plays a role in aberrant activation of NF-κB and, therefore, represents a potential therapeutic target.

INTRODUCTION

NF-κB[3]/Rel is a family of dimeric transcription factors distinguished by the presence of a 300-amino-acid region, termed the Rel homology domain (1). The NF-κB family includes five known members in mammals: p50/p105, p52/p100, c-Rel, RelB, and RelA (p65). Classical NF-κB complexes are composed of p50/RelA heterodimers. In most untransformed cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to the specific IκB-inhibitory proteins, of which IκB-α is the paradigm. A variety of agents that activate NF-κB, *e.g.*, TNF-α and interleukin 1, mediate degradation of IκB-α via a canonical pathway involving phosphorylation of IκB on two NH_2-terminal serine residues by a large multisubunit complex composed of the two IKKs IKKα and IKKβ (2, 3). This phosphorylation is followed by ubiquitination and rapid proteasome-mediated degradation of IκB, allowing for translocation of free NF-κB to the nucleus. In addition, phosphorylation of IκB by protein kinase CK2 (formerly casein kinase II) has been implicated in basal IκB-α degradation (4–8). CK2-mediated phosphorylation of IκB-α occurs preferentially at Ser-283, Ser-289, Thr-291, and Ser-293 within the COOH-terminal PEST domain, and mutations of these sites prolong IκB-α half-life (5, 7, 8). In lymphoid cells, an alternative calpain-mediated IκB-α degradation pathway was described that contributed to the constitutive NF-κB activation seen in these cells (9). Moreover, we demonstrated that CK2 phosphorylation accelerates calpain-mediated degradation of IκB in B cells (10). Thus, two alternative phosphorylation signals and proteolytic systems regulate degradation of IκB and activation of NF-κB in what appears to be a signal-and cell type-specific manner.

CK2 is a highly conserved serine/threonine kinase that recognizes the general consensus sequence (S/T)XX(D/E). CK2 is a constitutively active kinase that is ubiquitously expressed in both the cytoplasm and nucleus of eukaryotic cells and exists in cells as a holoenzyme containing two catalytic (CK2α or CK2α′) and two regulatory (CK2β) subunits. The two catalytic subunits are highly homologous, but CK2α′ has a unique required role in spermatogenesis (11). The crystal structure of human $CK2\alpha_2\beta_2$ was recently solved, showing flexible interdomain and intersubunit interactions in which each catalytic subunit makes no direct contact with the other and each interacts with both regulatory subunits, via the NH_2-terminal lobe of the catalytic subunit and an extended COOH-terminal tail of the regulatory subunit (12). CK2 is essential for cell viability, and many of the >160 CK2 substrates identified thus far are growth- and cell cycle-related (13). Overexpression or inhibition of CK2α, CK2α′, or CK2β subunits was shown to affect proliferation; however, results varied greatly with cell type (14–17). Overexpression of CK2 has been observed in many human cancers, including breast cancers (18–21). Furthermore, we have shown that enforced CK2α expression in transgenic mice is sufficient to induce T-cell lymphomas (22) and breast cancer (19).

We and others have demonstrated aberrant activation of NF-κB factors in breast cancer (23–27). High levels of nuclear NF-κB/Rel were found in human breast tumor cell lines and in the majority of primary human and rodent breast tumor tissue samples. In contrast, untransformed breast epithelial cells and normal mammary glands contain low basal levels of nuclear NF-κB/Rel. In breast cancer cells, elevated levels of NF-κB correlated with a decrease in the half-life of IκB-α protein (25). We observed that many primary breast tumor tissue samples and human cancer cells display an increase in either CK2 or IKK activity (20).

Recently, we have found that the Her-2/neu protein activates NF-κB via a PI3-K- to Akt kinase-signaling pathway that can be inhibited via the tumor suppressor PTEN (28). The *Her-2/neu* (or c-*erbB-2*) oncogene, the second member of the EGF receptor family (EGFR-2), encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu, which is seen in ~30% of breast cancers, is associated with poor overall survival, increased metastatic potential and resistance to chemotherapeutic agents (29). Surprisingly, degra-

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[3] The abbreviations used are: NF-κB, nuclear factor-κB; β-gal, β-galactosidase; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift analysis; GST, glutathione-*S*-transferase; PI3-K, phosphatidylinositol 3-kinase; WCE, whole cell extract; WT, wild type; MMTV, mouse mammary tumor virus; IKK, IκB kinase; TNF, tumor necrosis factor; CMV, cytomegalovirus; Oct1, Octomer-1 (oligonucleotide); PEST, proline-glutamic-serine-threonine rich region.

dation of IκB-α in the MMTV-Her-2/neu-derived mammary tumor cells NF639 (30) did not appear to be mediated via the IKK complex or the proteasome but, rather, was blocked on addition of calpain inhibitors (28). This raised the question of involvement of CK2 in activation of NF-κB in NF639 cells, which we have addressed here. Expression of kinase-inactive CK2 catalytic subunit mutants decreased basal NF-κB activity in NF639 cells, as well as in Hs578T human breast cancer cells, which were shown to overexpress CK2 (20). Furthermore, the inhibition of CK2 enhanced susceptibility to cell death and inhibited transformed phenotype of NF639 cells. Conversely, ectopic CK2 was sufficient to induce NF-κB activity in NIH 3T3 fibroblasts, which express low basal NF-κB and CK2 activities. These studies indicate that CK2 directly controls NF-κB activity, which, in turn, modulates the survival and transformed phenotype of breast cancers, including those in which *Her-2/neu* is amplified.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions. The NF639 MMTV-c-neu mouse mammary tumor cell line (kindly provided by P. Leder, Harvard Medical School, Boston, MA) was cultured as described previously (30). NMuMG is an untransformed, immortalized mouse mammary epithelial cell line and was cultured as described previously (31). The Hs578T human breast tumor cell line, which was derived from a carcinosarcoma and is epithelial in origin, was grown as described previously (24). NIH 3T3 mouse fibroblast, 293T human embryonic kidney cell line, and Phoenix packaging cell lines were cultured in DMEM supplemented with 10% FCS and penicillin-streptomycin (Invitrogen Life Technologies, Inc., Carlsbad, CA). For protein synthesis inhibition, exponentially growing cells were treated with 30–50 μg/ml cycloheximide (Sigma-Genosys Inc., The Woodlands, TX).

Transfection Conditions. For transfections, Fugene reagent was used according to the manufacturer's directions (Roche Diagnostics Corporation, Indianapolis, IN). To evaluate transcriptional activity, cells were transfected in triplicate with an NF-κB element-luciferase reporter vector, driven by three κB elements from upstream of the MHC class I promoter, kindly provided by A. Chan (Mt. Sinai School of Medicine, New York City, NY; Ref. 32). The SV40-promoter β-gal (pSV40-β-gal) reporter vector was used to normalize transfection efficiency. Luciferase assays were performed as described previously (33). Statistical analyses were performed using the Student's *t* test. The pSVK3-human IκB-α WT, 2N (S32A and S36A), 3C (S283A, T291A and T299A), and 2N3C (S32A, S36A, S283A, T291A, and T299A) plasmid vectors were a kind gift from J. Hiscott (Institut Lady Davis de Recherches Medicales; Ref. 5). The pRc/CMV2 HA-tagged CK2α (pZW2), pRc/CMV2-HA-CK2α K68M (pGP8), pRc/CMV2-HA-CK2α′ (pGP3), and pRc/CMV2-HA-CK2α′ K69M (pGP18) vectors, and the backbone empty vector were provided by D. W. Litchfield (University of Western Ontario, Ontario, Canada; Ref. 34). The CK2α K68M or CK2α′ K69M mutants contain a single point mutation in the kinase domain of CK2α and CK2α′ catalytic subunits, respectively, and are devoid of kinase activity (34). The pRc/CMV2 plasmid contains a *neo*-resistance gene driven by the CMV promoter (Invitrogen Life Technology). To establish stable transfectants, P100 dishes of cells were transfected with 20 μg of the appropriate pRc-CMV constructs. After 48 h, cells were selected with 600 μg/ml geneticin (Invitrogen Life Technology) for 10 days, and then grown in the presence of 100 μg/ml geneticin. Alternatively, cells were cotransfected with 1 μg of the pGKpuro selection plasmid, selected with 4 μg/ml puromycin (Sigma) for 4 days, and then grown in the presence of 1 μg/ml puromycin.

Retroviral Gene Delivery. The murine CK2α cDNA (22) was excised from the pcDNA3.0 vector (Invitrogen Life Technologies, Inc.) by *Bam*HI digestion and subcloned into the *Bam*HI site of the pBabe-puro ecotropic retroviral vector (35), yielding pBabe-puro-CK2α. Phoenix packaging cells were used for the generation of retrovirus, which were selected with 500 μg/ml hygromycin to increase Gag and Pol viral protein expression. Briefly, P100 dishes of 80% confluent Phoenix cells were transfected with 15 μg of pBabe-puro-CK2α or pBabe-puro along with 5 μg of an Env-expressing vector. After 24 h, the medium was changed. and cells were incubated for another 24 h at 32°C to increase retrovirus half-life. Supernatants containing retrovirus were then harvested, filtered and transferred on NIH 3T3 target cells in the presence of 2 μg/ml polybrene (Sigma). After 24 h, infected cells were washed, selected with complete medium plus 4 μg/ml puromycin for 4 days, and expanded in medium containing 1 μg/ml puromycin. Single cell clones were isolated by limiting dilution. As a positive control, cells were infected with the pBabe-puro-GFP retrovirus, indicating more than 90% efficiency in retroviral infection of NIH 3T3 cells (data not shown).

EMSA. The sequence of the WT URE NF-κB-containing oligonucleotide from the c-*myc* gene is as follows: WT, 5′-AAGTCCGGGTTTTCCCCAACC-3′ (36). The core element is underlined. The mutant URE has a two G-to-C-bp conversion, indicated in bold, which blocks NF-κB/Rel binding: 5′-AAGTCCGCCTTTTCCCCAACC-3′. The sequence of the Sp1 oligonucleotide is 5′-ATTCGATCGGGGCGGGGCGACC-3′. The Oct1 oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. Nuclear extracts from cell lines were prepared and samples (2.5–5 μg) subjected to EMSA as described (24). For antibody supershift analysis, the binding reaction was performed in the absence of the probe, the appropriate antibody was added, and the mixture incubated for 16 h at 4°C. The probe was then added and the reaction incubated an additional 30 min at 25°C and the complexes resolved by gel electrophoresis, as above. Antibodies used included: anti-RelA (C-20): sc-372, anti-p50 (NLS): sc-114, anti-p52 (K-27): sc-298; and anti-c-Rel (C): sc-71 (all from Santa Cruz Biotechnology, Santa Cruz, CA). When indicated, 250 ng of either IκB-α-GST fusion protein or GST alone or excess unlabelled WT or mutant oligonucleotide was added to the binding reaction just before addition of the probe. Data were quantified by densitometry using a Molecular Dynamics densitometer.

Immunoblotting. To prepare WCEs, cells were washed with PBS, resuspended in cold PD buffer [40 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.1% NP40]. Cells were lysed by sonication, and debris was removed by centrifugation. Samples of WCEs or nuclear extracts, prepared as described above, were separated by electrophoresis in polyacrylamide-SDS gels, transferred to polyvinylidine difluoride membrane (Millipore, Bedford, MA) and subjected to immunoblotting. Monoclonal antibodies specific for HA-tag (F7) and β-actin (AC-15) were purchased from Sigma. The rabbit polyclonal antibody specific for the CK2α subunit of human CK2 (residues 70–89) was from Stressgen (Victoria, British Columbia, Canada). Antibodies specific for mouse IκB-α (C-21), sc-371, and human IκB-α (C-15), sc-203, were from Santa Cruz Biotechnology. Antibodies specific for NF-κB subunits were the same as those used for the supershift assays.

CK2 Kinase Assay. Samples (2 μg) of WCEs were incubated with a 1 mM solution of the CK2-specific peptide substrate RRREEETEEE (Sigma-Genosys Inc.) in a CK2 kinase buffer {100 mM Tris (pH 8.0), 20 mM $MgCl_2$, 100 mM KCl, 0.1 μg/ml BSA, 100 μM Na_3VO_4, 5 μCi [γ-^{32}P]GTP} at 30°C for 10 min. The reaction was stopped by adding 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatmann filter and washed four times in 150 mM H_3PO_4 to remove unincorporated [γ-^{32}P]GTP; phosphorylated peptides were measured by scintillation counting. The samples were assayed in duplicate, and the background kinase activity in the absence of the peptide substrate was subtracted. For evaluation of phosphorylation of IκB-α, 10 to 20 μg of WCEs were diluted to 10-μl final volume in PD buffer. After the addition of 15 μl of buffer D {100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl, 20 mM $MgCl_2$, 100 μM Na_3VO_4, and 10 μCi [γ-^{32}P]GTP}, reactions were incubated at 30°C for 30 min in the presence of 200 ng GST-wtIκB-α as substrate. Alternatively, GST-3CIκB-α, with three point mutations at S283A, T291A, and T299A, kindly provided by J. Hiscott, was used as substrate (5). The kinase reaction was stopped by the addition of 2× SDS-PAGE sample buffer, and the mixture subjected to SDS-PAGE analysis and visualized by autoradiography. To evaluate the kinase activity of transfected WT or mutant proteins HA-CK2α or HA-CK2α′ in cell extracts, samples (100 μg) of WCEs were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech AB, Piscataway, NJ) for 1 h at 4°C. The HA-tagged CK2 proteins were collected using 1 μg of the HA-tag F7 antibody. Controls included immunoprecipitations performed with normal polyclonal mouse IgG (Santa Cruz Biotechnology). After 16 h of incubation and extensive washing, immunoprecipitates were subjected to a CK2 kinase assay, as described above.

TNF-α-induced Apoptosis Assay. Cells were plated at 2 × 10^4/ml in 96-well plates. After 24 h, cells were treated with recombinant human TNF-α

(PeproTech Inc., Rocky Hill, NJ) in the presence of 30 μg/ml cycloheximide (Sigma). After 16 h, cell viability was evaluated by the nonradioactive MTS cell proliferation assay (Promega, Madison, WI).

Focus Formation Assay. Cells were plated at 3×10^3/ml in top plugs consisting of complete medium and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, ME). Plates were subsequently incubated for 18 days in humidified incubator at 37°C. Cells were stained with 0.5 ml of 0.0005% crystal violet, and colonies were counted using a dissecting microscope.

RESULTS

Expression of Protein Kinase-inactive CK2α or CK2α′ Inhibits CK2 Activity in NF639 MMTV-Her-2/neu Mammary Tumor Cells. Using the NF639 cell line, which was established from a mammary tumor that arose in a MMTV-Her-2/neu transgenic mouse (30), we demonstrated that Her-2/neu activates classical NF-κB via a noncanonical pathway, *i.e.*, involving calpain- rather than IKK-induced proteasome-mediated degradation of IκB-α (28). Given the recent evidence implicating phosphorylation by CK2 in degradation of IκB-α via calpain (9, 10), we compared expression levels of CK2α catalytic subunit in NF639 cells with levels in untransformed NMuMG mouse mammary epithelial cells (Fig. 1*A*). As control for equal loading, samples were similarly analyzed for levels of β-actin (Fig. 1*A*). Immunoblot analysis revealed an increase in the level of CK2α protein in NF639 cells compared with NMuMG cells. Scanning of this and a duplicate blot analysis indicated that NF639 cells display a 2.8 ± 0.3-fold increase in total level of CK2α compared with NMuMG cells.

To inhibit constitutive CK2 activity in NF639 cells, vectors expressing kinase-inactive CK2α or CK2α′ subunits were used. Stably transfected pools of NF639 cells expressing HA-tagged HA-CK2α K68M or HA-CK2α′ K69M mutants were selected. As controls, cells were transfected with the pRc/CMV2 backbone vector, as well as HA-CK2α WT or HA-CK2α′ WT expression vectors. To examine the kinase activity of exogenously expressed CK2α and CK2α′, ectopic HA-tagged CK2 proteins were immunoprecipitated from WCEs with an HA-specific antibody, and CK2 kinase activity assayed as described previously (20), using as substrate either GST-wtIκB-α or GST-3CIκB-α (with S283A, T291A, and T299A point mutations in COOH-terminal residue targets of CK2 phosphorylation) as a negative control (Fig. 1*B*). In cells expressing HA-tagged CK2α WT or CK2α′ WT, HA-specific immunoprecipitations revealed substantial kinase activity. As expected, no kinase activity was detected with the mutant GST-3CIκB-α as a substrate, or with immunoprecipitates performed with normal mouse IgG. When kinase assays were performed on immunoprecipitates from cells transfected with the pRc/CMV2 backbone vector, no detectable kinase activity was observed. Immunoprecipitates from cells expressing HA-tagged CK2α K68M or CK2α′ K69M displayed less than 5% of the kinase activity seen with immunoprecipitates from cells expressing WT CK2 proteins. This small but reproducible kinase activity suggests that the transfected inactive subunits may form holoenzyme complexes with endogenous WT CK2α- or CK2α′-active subunits. To examine the effect of the kinase-inactive variants on total CK2 activity, we performed a kinase assay on WCEs with the CK2-specific peptide substrate RRREEETEEE (Fig. 1*C*). We observed that NF639 cells expressing CK2α K68M or CK2α′ K69M displayed an ~30–40% drop in total CK2 activity. Therefore, expression of CK2α K68M or HA-CK2α′ K69M resulted in a partial inhibition of constitutive CK2 activity in NF639 cells.

Inhibition of CK2 Reduces Basal NF-κB Binding Activity and Stabilizes IκB-α Degradation in Her-2/neu-induced NF639 Breast Cancer Cells. We next measured the effects of CK2 inhibition on the Her-2/neu-induced NF-κB activity in NF639 cells, which we described previously (28). To assess NF-κB binding levels, nuclear extracts were prepared from NF639 cells expressing HA-CK2α K68M, HA-CK2α′ K69M, or both kinase-inactive variants combined, and were subjected to EMSA. As probe, an oligonucleotide containing the NF-κB element upstream of the c-*myc* promoter was used and Oct1 served as a control for equal loading (Fig. 2*A*). Two major NF-κB complexes were observed with extracts from the NF639 cell line, which have been identified previously as p50/RelA and p50 homodimer complexes (28). Expression of kinase-inactive CK2α or kinase-inactive CK2α′ caused a significant reduction in both p50/RelA and p50/p50 binding levels compared with transfection with the pRc/CMV2 plasmid. Coexpression of CK2α K68M and CK2α′ K69M mutants did not have an additive effect.

To further study the effect of inhibition of CK2 on cellular localization of NF-κB subunits, immunoblot analysis was performed for RelA, p50, and p52 NF-κB subunits in WCEs and nuclear extracts from CK2α K68M-expressing NF639 cells or pRc/CMV2-transfected control cells (Fig. 2*B*). Results showed that nuclear levels of p50 and RelA were significantly lower in CK2α K68M-expressing cells compared with the control cells, whereas total levels were increased in the cells, presumably localized in the cytoplasm. In contrast, no changes

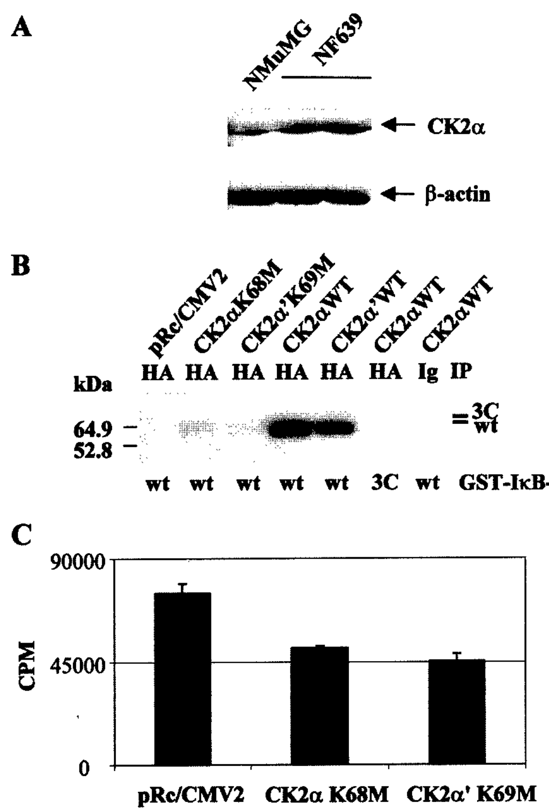

Fig. 1. Expression of kinase-inactive CK2α or CK2α′ subunit inhibits CK2 activity in Her-2/neu-induced NF639 breast cancer cells. *A*, CK2α expression. WCEs were prepared from two independent cultures of NF639 and NMuMG untransformed mouse breast epithelial cells at 70% confluence. Samples (80 μg) were subjected to immunoblot analysis for CK2α and β-actin levels. *B*, expression of transfected CK2 constructs in NF639 cells. Cells were transfected with parental pRc/CMV2, HA-CK2α K68M, HA-CK2α′ K69M, HA-CK2α WT, or HA-CK2α′ WT plasmid expression vectors. Stably transfected cells were selected with puromycin. WCEs were prepared, and equal amounts (300 μg) were immunoprecipitated with either the F7 antibody against HA-tag (HA) or normal mouse IgG (*Ig*). Immunoprecipitates were subjected to a CK2 kinase assay in the presence of [γ-^{32}P]GTP using either GST-wtIκB-α or, as a negative control, GST-3CIκB-α (*3C*, with three point mutations at S283A, T291A, and T299A in the COOH-terminal PEST domain). Proteins were resolved by PAGE-SDS and visualized by autoradiography. Positions of molecular weight protein standards of M_r 64,900 (*64.9*) and 52,800 (*52.8*) and of the GST-wtIκB-α (*wt*) and GST-3CIκB-α (*3C*) proteins are as indicated. *C*, total CK2 activity. NF639 cells were transfected with the indicated constructs and selected with geneticin. Samples of WCEs (2 μg) were assayed in duplicate for CK2 phosphorylation using a specific CK2 substrate peptide. Data are expressed as the means with background (cpm in the absence of peptide) subtracted.

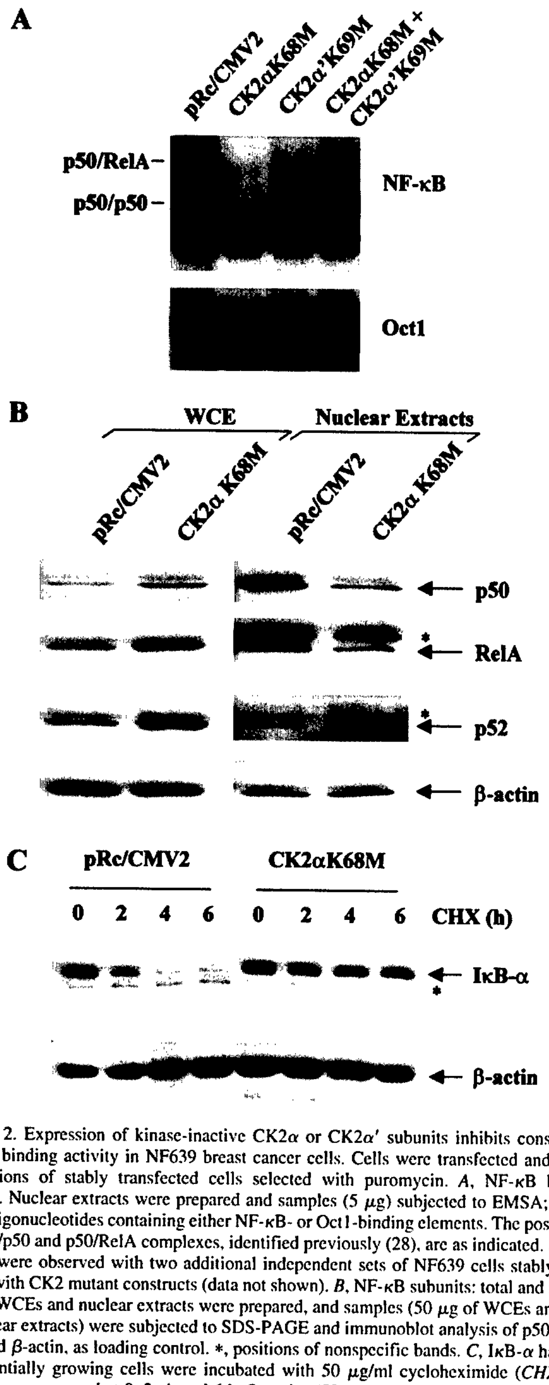

Fig. 2. Expression of kinase-inactive CK2α or CK2α′ subunits inhibits constitutive NF-κB binding activity in NF639 breast cancer cells. Cells were transfected and mixed populations of stably transfected cells selected with puromycin. *A*, NF-κB binding activity. Nuclear extracts were prepared and samples (5 μg) subjected to EMSA; probes were oligonucleotides containing either NF-κB- or Oct1-binding elements. The position of the p50/p50 and p50/RelA complexes, identified previously (28), are as indicated. Similar results were observed with two additional independent sets of NF639 cells stably transfected with CK2 mutant constructs (data not shown). *B*, NF-κB subunits: total and nuclear levels. WCEs and nuclear extracts were prepared, and samples (50 μg of WCEs and 5 μg of nuclear extracts) were subjected to SDS-PAGE and immunoblot analysis of p50, RelA, p52, and β-actin, as loading control. *, positions of nonspecific bands. *C*, IκB-α half-life. Exponentially growing cells were incubated with 50 μg/ml cycloheximide (*CHX*), and WCEs were prepared at 0, 2, 4, and 6 h. Samples (50 μg) were subjected to immunoblot analysis for IκB-α, and β-actin, as loading control. *, the position of a nonspecific band.

could be detected in nuclear p52 levels. Equal loading was confirmed by an analysis of β-actin levels. Therefore, the inhibition of CK2 results in reduced NF-κB binding caused by decreased nuclear levels of RelA and p50 proteins.

CK2-mediated phosphorylation of IκB-α in the COOH-terminal PEST domain has been implicated in the basal and signal-dependent turnover of free and NF-κB-bound IκB-α (4–8). We next tested the involvement of CK2-mediated phosphorylation in the rate of IκB-α turnover in NF639 cells. Exponentially growing NF639 cells, stably transfected with HA-CK2α K68M or parental vectors, were treated with the protein synthesis inhibitor cycloheximide and WCEs prepared at the times indicated and subjected to immunoblotting for IκB-α expression (Fig. 2*C*). The stability of IκB-α protein was substantially increased in NF639 cells upon inhibition of CK2. In two independent sets of stably transfected NF639 cells, the half-life of IκB-α decay was between ~2 and 4 h in pRc/CMV2-transfected control cells and increased to more than 6 h in cells expressing CK2α K68M (Fig. 2*C*, and data not shown). Overall, these results demonstrate the ability of protein kinase CK2 to affect NF-κB levels in Her-2/neu-induced NF639 breast cancer cells via the regulation of IκB-α phosphorylation and degradation.

Inhibition of Protein Kinase CK2 Sensitizes Her-2/neu-induced NF639 Mouse Breast Cancer Cells to Apoptosis and Loss of Anchorage-independent Growth. Work from many laboratories, including our own, have highlighted the importance of constitutive NF-κB activity in protecting breast cancer cells from apoptosis (24). We, therefore, assessed the effect of the inhibition of CK2 on TNF-α-induced cell death. NF639 cells, stably transfected with HA-CK2α K68M expression vector, were treated with cycloheximide and stimulated with an increasing dose of TNF-α (25–400 ng/ml). Cell viability was assessed by MTS assay 24 h after stimulation. As a control, NF639 cells, stably expressing the IκB-α 2N3C (S32A, S36A, S283A, T291A, and T299A) super-repressor mutant, which cannot be phosphorylated by IKK or CK2 kinases and is, therefore, resistant to degradation, were similarly assessed. NF639 cells that expressed IκB-α 2N3C displayed a profound decrease in p50/RelA binding and NF-κB transcriptional activity, as expected (data not shown). Minimal cell death was observed in parental pRc/CMV2-transfected NF639 cells, which is consistent with the elevated NF-κB constitutive levels observed in this cell line (Ref. 28; Fig. 3*A*). In contrast, cells expressing CK2α K68M or IκB-α 2N3C displayed increased susceptibility to TNF-α-induced cell death, as judged by MTS assay. Results obtained with this and a second independent set of stably transfected cells were quantified. At a concentration of 400 ng/ml TNF-α, the percentage of viable pRc/CMV2-, CK2α K68M-, or IκB-α 2N3C-expressing NF639 cells was 85.0 ± 5.6%, 52.5 ± 4.9% ($P < 0.001$), and 58.5 ± 12.1% ($P < 0.03$), respectively. Therefore, the inhibition of CK2 activity sensitizes NF639 cells to TNF-α-induced cell death.

We next asked whether the inhibition of CK2 affected the transformed phenotype of NF639 breast cancer cells. Cultures of two independent sets of NF639 cells stably expressing kinase-inactive CK2α were assessed for growth in soft agar (Fig. 3*B*). The numbers of colonies per high power field with the parental pRc/CMV2 *versus* CK2α K68M vectors were as follows: 124 ± 20 and 37 ± 18 ($P < 0.01$, first set of cell populations), and 107 ± 13 and 38 ± 6 ($P < 0.002$, second set of cell populations). Thus, the inhibition of CK2 activity leads to a substantial reduction in the transformed phenotype of Her2/neu breast cancer cells, as measured by the loss of anchorage-independent growth of these cells.

Expression of Kinase-inactive CK2α Inhibits NF-κB Activity in Hs578T Human Breast Cancer Cells and 293T Human Embryonic Kidney Cells. We next asked whether the inhibition of CK2 activity in other cell lines would similarly decrease NF-κB activity. The Hs578T human breast tumor cell line, which overexpresses CK2 protein and kinase activity (20), was selected for study. Hs578T cells were identified previously to express predominantly activated p50/RelA and p50/p50 NF-κB complexes (20, 24). For these experiments, mixed cell populations of Hs578T cells stably expressing HA-tagged mutant CK2α subunit were obtained by geneticin selection. Nuclear extracts and WCE were prepared and analyzed for NF-κB binding levels and expression of HA-tagged CK2, respectively (Fig. 4*A*). Results obtained with this and a second set of stably transfected cells showed an ~50% drop in NF-κB binding in HA-CK2α K68M-expressing Hs578T cells compared with parental pRc/CMV2-transfected cells. Expression of CK2α K68M mutant was confirmed by

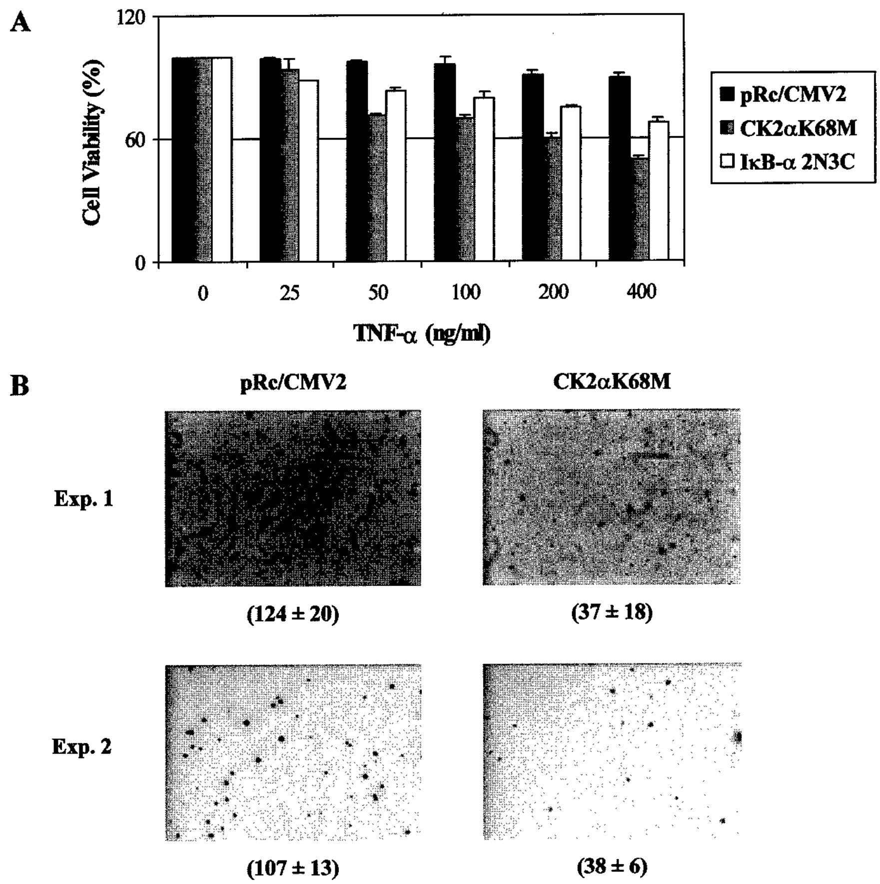

Fig. 3. Expression of kinase-inactive CK2α subunit increases susceptibility to TNF-α-induced apoptosis and reduces anchorage-independent growth of NF639 breast cancer cells. *A*, TNF-α-induced cell death. Mixed population of NF639 cells stably transfected with HA-CK2α K68M, parental pRc/CMV2, or IκB-α 2N3C super-repressor plasmid expression vectors were plated at 2×10^3 in 96-well plates. After 24 h, cells were treated with recombinant human TNF-α in the presence of 30 μg/ml cycloheximide. After 16 h, cell viability was evaluated by the MTS cell proliferation assay. Results are expressed as percentage of viable cells (absorbance at 490 nm normalized to absorbance of cultures treated with cycloheximide alone). *B*, soft agar assay. Cells were plated, in triplicate, at 3×10^3/ml in top plugs consisting of complete medium and 0.4% agarose. After 18 days, the numbers of foci were scored and pictures taken with a Nikon camera (×1.5). Results were obtained with two independent sets of transfected cells. *Below the images*, values of colony number per high power field.

immunoblotting for the HA tag. Thus, the inhibition of CK2 similarly reduces NF-κB binding in Hs578T human breast cancer cells.

To assess whether the regulation of CK2 activity could be extended to another cell type, 293T human embryonic kidney cells were similarly tested. Because transfection of these cells occurs with a high efficiency, transient transfections were performed with either HA-CK2α K68M or HA-CK2α′ K69M expression vectors or the parental vector, as control. Cells were harvested 6 days after transfection to ensure substantial expression of ectopic proteins compared with endogenous proteins. As seen in Fig. 4*B*, expression of kinase-inactive CK2α or CK2α′ mutants resulted in significant decreases in NF-κB binding. An 89% and 76% drop in band 1 and band 2, respectively, was noted in 293T cells expressing CK2α′ K69M compared with parental cells. In 293T cells, the CK2α′ K69M was found to have a stronger effect than CK2α K68M, consistent with the higher expression level of transiently expressed CK2α′ mutant compared with CK2α mutant that was routinely seen (Fig. 4*B*, bottom panel, and data not shown). These results extend the finding on the ability of kinase-inactive forms of CK2 to reduce constitutive NF-κB levels to 293T human embryonic kidney cells.

CK2α Overexpression in NIH 3T3 Cells by Retroviral Gene Delivery Leads to Increased Nuclear NF-κB Expression. To determine whether increased CK2 expression is sufficient to induce NF-κB levels, we next attempted to increase CK2 activity through overexpression of the CK2α catalytic subunit of CK2. We turned to the NIH 3T3 fibroblast cell line, which has lower levels of endogenous CK2 than the NF639 or Hs578T cell lines (data not shown). Cells were retrovirally infected with a vector expressing an untagged murine CK2α, and a mixed population of infected cells as well as single clones were selected in puromycin and were screened for total CK2α protein levels. Two stable CK2α-overexpressing NIH 3T3 clones, designated Clone 4 and Clone 6, were chosen for this study. To monitor the relative levels of CK2α expression in the selected cells, immunoblot analysis was performed with a CK2α polyclonal antibody using samples of WCEs and nuclear protein extracts (Fig. 5*A*). Two bands were detected in the WCEs. The top band corresponded to the full length CK2α (M_r ~45,000), and the bottom one to a protein of M_r ~40,000. Immunoblot analysis performed with antibodies specific for either the NH_2-terminal or COOH-terminal part of CK2α indicated that the M_r ~40,000 protein likely resulted from COOH-terminal clipping of CK2α (data not shown), as described previously during the *in vitro* purification of human recombinant CK2α (12). The WCEs of the Babe-CK2α mixed population, Clone 4, and Clone 6 demonstrated elevated levels of CK2α protein compared with the parental vector control (Babe)-infected NIH 3T3 cells, which showed only low basal expression. When the blots were scanned and normalized to β-actin levels, a 2.6-, 15.1-, and 9.5-fold increase in CK2α expression was observed in NIH 3T3 Babe-CK2α mixed-population cells, Clone 4, and Clone 6, respectively, compared with the parental Babe cells. No significant increase in CK2α′ was observed in Babe-CK2α cells (data not shown). Higher levels of CK2α expression were also detected in the nuclei of Babe-CK2α cells (Fig. 5*A*). These results were confirmed by immunofluorescent staining of CK2α, which showed a strong accumulation of CK2α in both the cytoplasm and the nuclei of Clone 4 and Clone 6 compared with parental NIH 3T3 Babe cells (data not shown). To confirm that increased CK2α expression led to increased CK2 enzymatic activity, a CK2 kinase assay was performed with GST-wtIκB-α or GST-3CIκB-α as substrates. WCEs, prepared from NIH 3T3 Babe-CK2α mixed population and clones, were used directly in *in vitro* CK2 phosphorylation assays (Fig. 5*B*). Kinase assays demonstrated strong preferential phosphorylation of GST-wtIκB-α compared with GST-3CIκB-α, consistent with the assay measuring CK2 activity. The mixed population, Clone 4, and Clone 6 displayed an increase in CK2 kinase

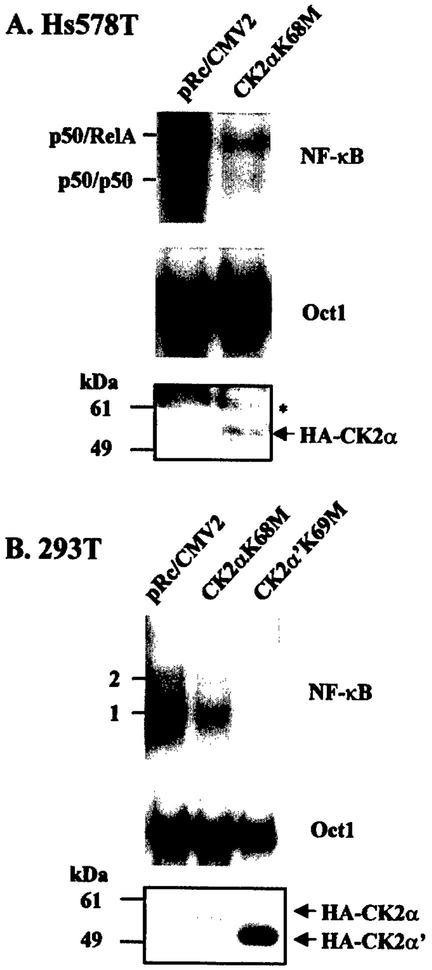

Fig. 4. Expression of kinase-inactive CK2α or CK2α' subunit inhibits NF-κB binding activity in Hs578T human breast cancer cells and 293T human embryonic kidney cells. *A*, Hs578T cells. Mixed population of cells, stably transfected with pRc/CMV2 or HA-CK2α K68M plasmid vectors, were selected in geneticin, and nuclear extracts and WCEs were prepared. *Top panel*, samples of nuclear extracts (5 μg) were subjected to EMSA for NF-κB or Oct1. The position of the p50/p50 and p50/RelA complexes identified previously (20) are as indicated. *Bottom panel*, samples of WCE (80 μg) were subjected to immunoblot analysis of exogenous CK2α, using an HA tag-specific antibody. *, the position of a nonspecific band. The position of molecular weight protein standards of M_r 61,000 (*61*) and M_r 49,000 (*49*) are as indicated. *B*, 293T cells. Cells were transiently transfected with parental pRc/CMV2, HA-CK2α K68M, or HA-CK2α' K69M plasmid vectors. Cells were harvested after 6 days and analyzed as described in *A*. *Band 1* and *band 2*, the two major NF-κB complexes.

activity of 1.2-, 2.3-, and 1.6-fold, respectively, compared with the parental NIH 3T3 Babe cells. Although the magnitude of increase in activity is less than the increase in protein, these results confirm that the two clones and the mixed population of cells display elevated CK2 activity.

We next assessed NF-κB binding levels by EMSA (Fig. 6). Nuclear extracts from the parental NIH 3T3 Babe cells displayed low levels of two NF-κB binding complexes, as observed previously (37). NIH 3T3 BabeCK2α mixed population of cells displayed increased levels of the two NF-κB complexes (Fig. 6*A*). Higher levels of these complexes were detected in both the Clone 4 and Clone 6 cells. When these results were scanned, a 1.5- and 2.5-fold increase in band 1 and band 2, respectively was noted in Clone 4 cells, and a 1.7- and 2.9-fold increase in band 1 and band 2, respectively, was noted in Clone 6 cells, compared with parental Babe cells. To determine the composition of NF-κB complexes, antibody supershift analysis was performed using extracts from Clone 4 cells (Fig. 6*B*). The major NF-κB complexes appeared to consist of p50/RelA heterodimers (Fig. 6*B*, *band 2*) and homodimers of p50 (Fig. 6*B*, *band 1*). No binding of other NF-κB subunits such as p52 or c-Rel was seen. Similar results were obtained with the parental Babe cells (data not shown). Successful competition with WT but not mutant oligonucleotide and inhibition upon addition

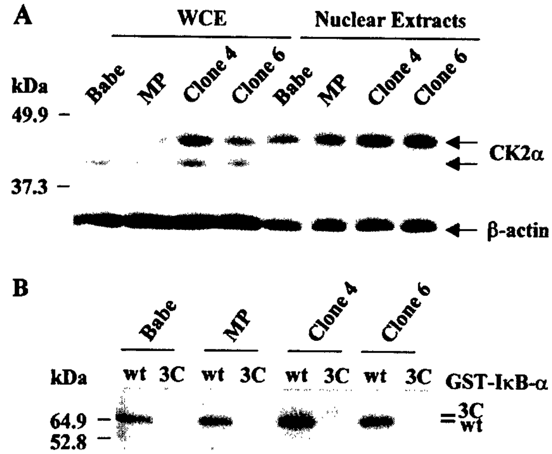

Fig. 5. Overexpression of the CK2α subunit in NIH 3T3 cells. NIH 3T3 cells were infected with recombinant retrovirus for pBabe (*Babe*) or pBabe-CK2α vectors. A mixed population of Babe-CK2α (*MP*) cells and two clones (*Clone 4* and *Clone 6*) were selected in puromycin. *A*, CK2α expression. Samples of WCEs (80 μg) or nuclear extracts (15 μg) from the indicated cells were separated by SDS-PAGE and subjected to immunoblot analysis for CK2α and β-actin levels. Two bands are seen in the WCEs with the CK2α antibody. *Top band*, the full-length CK2α protein; *bottom band*, (likely) a COOH-terminal clipped form of CK2α. *B*, CK2 activity. WCEs were prepared from the indicated cell populations, and samples (10 μg) were subjected to a CK2 kinase assay using as substrate either GST-wtIκB-α (*wt*) or GST-3CIκB-α (*3C*), as a negative control.

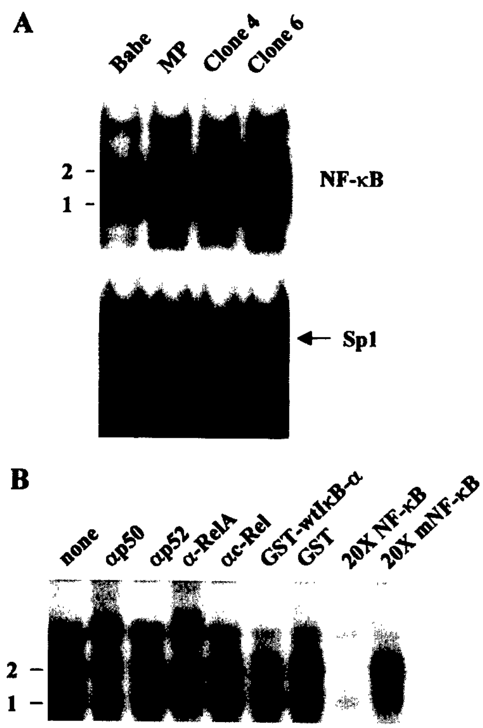

Fig. 6. Overexpression of CK2α subunit results in increased NF-κB binding in NIH 3T3 cells. *A*, NF-κB binding activity. Nuclear extracts were prepared from the indicated cells, and samples (5 μg) were subjected to EMSA with oligonucleotides specific for NF-κB and Sp1. *Band 1* and *band 2*, the positions of the two major NF-κB complexes. *B*, supershift analysis. Nuclear extracts from NIH 3T3 Babe-CK2α Clone 4 cells were incubated in the absence (*none*), or presence of antibody (α) against p50, p52, RelA, or c-Rel NF-κB subunits, or 1 μg GST-wtIκB-α or GST protein, and were subjected to EMSA with a NF-κB oligonucleotide. To test for binding specificity, the binding reaction was performed in the presence of 20× excess unlabelled WT (*20X NF-κB*) or mutant (*20X mNF-κB*) NF-κB oligonucleotide.

of GST-wtIκB-α confirmed the specificity of the NF-κB binding (Fig. 6*B*). Thus, overexpression of CK2α in NIH 3T3 cells leads to up-regulation of classical NF-κB binding.

CK2α Overexpression in NIH 3T3 Cells Leads to Increased Turnover of IκB-α and NF-κB Transcriptional Activity. To determine whether the increase in NF-κB binding upon overexpression of CK2α is caused by increased IκB-α degradation, exponentially growing NIH 3T3 Babe-CK2α Clone 4, Clone 6 and parental Babe cell lines were incubated in the presence of cycloheximide for a period of 0, 4, 8, or 12 h. WCEs were isolated and subjected to immunoblotting for IκB-α expression (Fig. 7*A*, *left panel*). The results were scanned, and the half-life of decay of IκB-α protein was >12 h in parental Babe cells, whereas it was 8.1 and 5.9 h in Babe-CK2α Clone 4 and Clone 6 cells, respectively (Fig. 7*A*, *right panel*). In this and two duplicate experiments, IκB-α protein decayed with a $t_{1/2}$ of 10.2 ± 0.3 h, 5.4 ± 1.4 h ($P < 0.01$), and 6.0 ± 0.3 h ($P < 0.001$) in parental Babe, Clone 4, and Clone 6 cells, respectively. Thus, IκB-α is more rapidly degraded in the CK2-overexpressing cells. To confirm the involvement of CK2 in enhanced IκB-α degradation, Babe-CK2α clones were transfected with human IκB-α WT, 2N (S32A, S36A) mutant or 3C (S283A, T291A, and T299A) mutant in plasmid vectors. The half-lives of IκB-α WT and 2N proteins were found to be quite similar, and much shorter than that of IκB-α 3C mutant, which cannot be phosphorylated by CK2 (data not shown), consistent with the rate of decay of IκB-α protein dependent on CK2-phosphorylation.

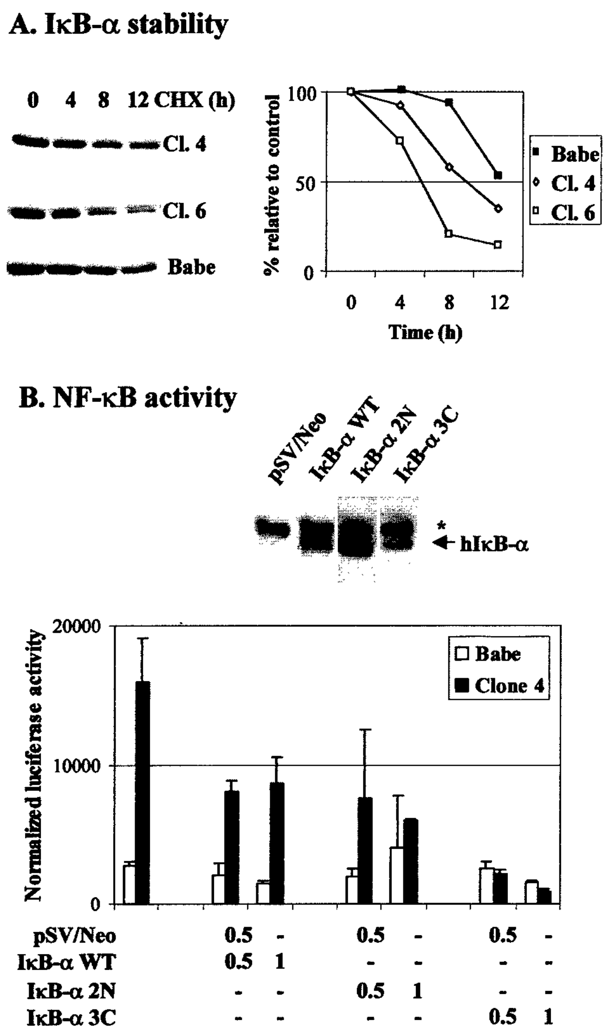

Fig. 7. Overexpression of CK2α subunit results in a higher rate of IκB-α degradation and NF-κB transcriptional activity in NIH 3T3 cells. *A*, IκB-α half-life. *Left panel*, the indicated cells, in exponential growth, were treated with 50 μg/ml cycloheximide (*CHX*), and WCEs were prepared at 0, 4, 8, or 12 h. Samples (50 μg) were subjected to immunoblot analysis for IκB-α. *Right panel*, the immunoblots in the left panel were subjected to densitometry, and the results were presented as the percentage relative to the control. *B*, NF-κB transcriptional activity. *Lower panel*, cultures of parental Babe or Clone 4 cells were transiently transfected, in triplicate, with 1 μg of NF-κB element-driven luciferase reporter construct and 0.5 μg of pSV40-β-gal in the presence of 0.5 or 1 μg of pSV/Neo empty parental vector or of the human IκB-α construct vectors: WT (*WT*), 2N mutant (S32A, S36A), or 3C mutant (S283A, T291A, and T299A in the COOH-terminal PEST domain). After 48 h, cultures were harvested, normalized for β-gal activity, and assayed for luciferase activity. Values in the bar graph, luciferase normalized for β-gal activity. *Top panel*, Babe-CK2α Clone 4 cells were transfected with equal amounts of pSV/Neo empty parental vector or human IκB-α construct vectors: WT, 2N mutant, or 3C mutant. After 18 h, WCEs were prepared, and samples (50 μg) were subjected to immunoblot analysis for human IκB-α (*hIκB-α*) using a human-specific antibody. *, the position of a nonspecific band. (All lanes are from the same gel.)

We next assessed the effect of overexpression of CK2α on NF-κB transcriptional activity, comparing parental Babe and Babe-CK2α Clone 4 cells. The cells were transfected with vectors expressing a NF-κB element luciferase reporter plus pSV40-β-gal, for normalization (Fig. 7*B*). Clone 4 cells displayed an ~5.5-fold increase in NF-κB transcriptional activity compared with parental cells. To confirm that CK2-mediated activation of NF-κB transcriptional activity is dependent on COOH-terminal PEST phosphorylation and degradation of IκB-α, cells were transfected with 0.5 or 1 μg of the IκB-α 3C mutant (S283A, T291A, and T299A), the IκB-α WT, or the IκB-α 2N mutant (S32A, S36A). The IκB-α 3C was much more effective at inhibiting NF-κB transcription in Clone 4 cells than were the WT IκB-α or the IκB-α 2N mutant, which cannot be phosphorylated by the IKKs. At the higher dose of plasmid transfection, expression of the IκB-α 3C mutant resulted in a 95% decline in NF-κB activity, whereas expression of IκB-α WT or IκB-α 2N mutant caused a drop of 46 and 62%, respectively, in NF-κB transcriptional activity. Western blot analysis indicated that levels of IκB-α 3C were lower or comparable with those of IκB-α WT or IκB-α 2N after transient transfection in Clone 4 cells (Fig. 7*B*). Thus, these results confirm that phosphorylation of IκB-α in the COOH-terminal PEST domain is physiologically relevant in regulating NF-κB activity in these cells.

DISCUSSION

Here we show that the inhibition of elevated CK2 activity in cancer cells reduces constitutive NF-κB activity, whereas ectopic expression of CK2 is sufficient to induce NF-κB activity in NIH 3T3 fibroblasts. Thus, CK2 plays a pivotal role in the regulation of constitutive NF-κB activity. Importantly, we show for the first time that the inhibition of CK2 activity decreases Her-2/neu-induced NF-κB activity. Thus, kinase-inactive CK2α subunit decreased nuclear NF-κB and transformed phenotype, whereas it increased sensitivity to TNF-α induced death in MMTV-Her-2/neu-derived NF639 mouse mammary carcinoma cells. Similarly, the inhibition of CK2 in Hs578T cells, which also display elevated basal CK2 activity (20), decreased NF-κB binding. Conversely, CK2α overexpression in NIH 3T3 cells was sufficient to increase IκB-α turnover and basal NF-κB activity. Previously, we and others demonstrated that primary breast cancer samples from patients or from a carcinogen-induced rodent model as well as breast cancer cell lines display increased CK2 activity (18–21) and aberrant activation of NF-κB (23, 24, 26, 27), whereas only low levels of CK2 and NF-κB activation were detected in normal breast epithelial cells. Our findings here demonstrate a direct link between overexpression of CK2 and NF-κB in these cancers. Furthermore, they suggest that CK2 kinase is a downstream mediator of Her-2/neu signaling and, thus, represents a potential new therapeutic target for the treatment of these malignancies.

In our studies, we made use of CK2α K68M or CK2α′ K69M mutants, which display a single point mutation in the kinase domain of these catalytic subunits and which are devoid of kinase activity (34). In the breast cancer cells, these inactive subunits were capable of reducing CK2 activity, *i.e.,* acting as dominant negatives, although they do not act this way in all cells (Ref. 16; as discussed below). In particular, we observed a 30–40% inhibition of total CK2 activity in NF639 cells expressing CK2α K68M or CK2α′ K69M as compared with parental cells. The inhibition of CK2 resulted in a drop in NF-κB binding in both NF639 and Hs578T breast tumor cells as well as in 293T human embryonic kidney cells. Thus, CK2 inhibition had a direct affect on NF-κB activity in various cell types. Consistent with these observations, we observed previously that treatment with the selective pharmacological inhibitors of CK2, apigenin or emodin, inhibited NF-κB activity in human breast cancer cell lines (20), and in mouse B-cell lymphomas (10). Interestingly, whereas ectopic expression of WT mouse CK2α catalytic subunit in NIH 3T3 fibroblast cells, which display low basal CK2 activity, led to a substantial increase in CK2 activity and NF-κB binding and activity, we were unable to similarly increase levels of CK2 protein or activity in NF639 cells (data not shown). Interestingly, the NF-κB activity in CK2α-overexpressing NIH 3T3 cells consisted predominantly of classical p50 and RelA-containing complexes, similar to breast tumor tissue from transgenic mice overexpressing CK2α in the mammary gland (19).

We observed previously that Her-2/neu activates NF-κB via a PI3-K-to-Akt-kinase signaling pathway that can be inhibited via antibody against the receptor or by the tumor suppressor PTEN in NF639 breast cancer cells (Ref. 28; see Fig. 8). Different mechanisms may be involved in the regulation of NF-κB activity by CK2. Previous work has indicated that basal and signal-dependent turnover of free and NF-κB-bound IκB-α is controlled by phosphorylation of residues in the COOH-terminal PEST domain by CK2, and, thus, mutation of these sites results in longer half-life of the IκB protein (5, 7, 8). Our evidence indicates that the level of CK2 activity affects IκB-α stability in NF639 cells (Fig. 8). Previously, we demonstrated that the dominant proteolytic pathway for IκB-α degradation in NF639 cells is mediated via calpain (28); and, because in B cells, we observed that CK2 phosphorylation accelerates degradation of IκB by calpain (10), it is possible that a similar mechanism occurs in breast cancer cells. The mechanisms leading to enhanced CK2 activity and the potential role of any additional kinases in the signaling pathway remain to be determined (Fig. 8). In addition, CK2 has been proposed to control NF-κB transcriptional activity by direct phosphorylation of the RelA subunit in response to TNF-α stimulation (38, 39). Here, we observed that NF-κB in CK2α-overexpressing NIH 3T3 cells is clearly nuclear and transcriptionally active in a reporter assay; however, it remains to be determined whether CK2 has a similar affect on basal activity of RelA in breast cancer cells. In addition, CK2 phosphorylates PTEN directly (40–42). Phospho-PTEN has increased stability but reduced lipid phosphatase activity (40), thereby promoting Akt phosphorylation and activation (42). However, no significant change in the activity of Her-2/neu receptor or in the levels of phosphorylated Akt was evident in NF639 breast cancer cells transduced with the kinase-inactive CK2 subunits (data not shown), which suggests that either PTEN expression itself is down-regulated or PTEN phosphorylation cannot be reduced by the kinase-inactive CK2 constructs.

CK2 has been reported to affect cell growth and transformation. Dysregulated expression of both CK2α and CK2α′ affect cell proliferation, transformation, and survival; although, the effects of ectopic CK2 subunit expression appear to depend on the type of cells used. For instance, inducible transient expression of kinase-inactive CK2α′ K69M in the presence of ectopic CK2β led to a strong attenuation of proliferation in the human osteosarcoma U2-OS cell line, although it did not inhibit total CK2 activity (16). Li *et al.* (15) reported that expression of ectopic Myc-tagged CK2α increased total CK2 activity and moderately

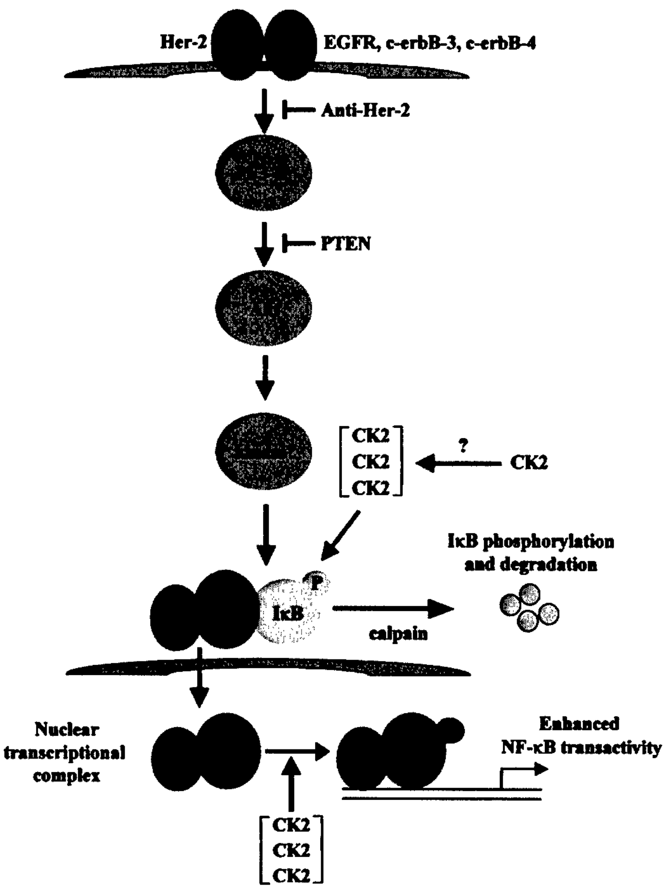

Fig. 8. Scheme of CK2 involvement in Her-2/neu-mediated NF-κB activation in breast cancer cells. Overexpression of Her-2/neu and heterodimerization with other EGF receptor family members (*EGFR, c-erbB-3, c-erbB-4*) activate NF-κB via a PI3-K-to-Akt-kinase signaling pathway that can be inhibited via treatment with an anti-Her-2/neu antibody or the tumor suppressor PTEN (28). Increased cytoplasmic and nuclear CK2 expression and activity have been observed in NF639 cells and many human breast cancers, although the mechanisms controlling this increase remain to be elucidated. Overexpression of CK2 leads to increased IκB-α phosphorylation and rate of degradation, which we have shown to be mediated via a calpain proteolytic pathway (28). The possibility that elevated levels of CK2 promotes NF-κB transcriptional activity by direct phosphorylation of its RelA subunit, as seen previously on TNF-α treatment (38, 39), is under investigation in breast cancer cells.

enhanced the growth of Chinese hamster ovary cells, whereas there was only a low level of expression of the ectopic protein, and no change in growth was seen in 3T3 L1 cells. Expression of kinase-inactive CK2α K68A was also reported to impair cell proliferation in both NIH 3T3 and CCL39 fibroblastic cells, and was linked to a defect in G_1-S phase progression (17). The inhibition of CK2α by antisense oligodeoxynucleotides induced apoptosis in human squamous cell carcinoma of the head and the neck (43). In contrast, overexpression of active forms of CK2α or CK2α′ had little or no detectable effect on the proliferation of these cells (16, 17); although, they did cooperate with Ha-*ras* in the transformation of rat embryo fibroblasts or BALB/c 3T3 fibroblasts (14). We observed that stable transfection of NF639 breast cancer cells with CK2α K68M only marginally affected cell proliferation, similar to the effect of stable expression of IκB-α super-repressor mutant (data not shown). Of note, expression of CK2α K68M significantly inhibited colony formation in soft agar. Furthermore, CK2α K68M-expressing cells displayed increased susceptibility to TNF-α-mediated cell death, which is tightly controlled by NF-κB. Together, these results suggest a key role of CK2 in the control of transformed phenotype and cell survival.

Aberrant nuclear NF-κB activity has been reported in many cancers (44). Products of several oncogenes such as *Her-2/neu* (28, 45), the EGF receptor signaling pathway (27, 46), and the oncogenic Raf and

Ras proteins (47, 48) induce NF-κB activity in various cell types. CK2 is markedly elevated in a number of hematopoietic and solid tumors (49) and, given our findings that demonstrate the ability of CK2 to directly affect NF-κB activation, it would be interesting to determine whether NF-κB and CK2 can cooperate to induce transformation. Experiments are in progress with bitransgenic mice that overexpress the c-Rel NF-κB family member and CK2α subunit in mammary epithelial cells. Lastly, our findings suggest that combinations of proteasome and calpain inhibitors or IKK and CK2 kinase inhibitors could be more effective than the use of single inhibitors in blocking IκB degradation and NF-κB activation, in promoting tumor cell apoptosis, and in sensitizing cancer cells to the proapoptotic effects of radiation or chemotherapy.

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